Characterization of photoautotrophic picoplankton assemblages in turbid, alkaline lakes of the Carpathian Basin (Central Europe)

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ABSTRACT

The photoautotrophic picoplankton (PPP) of ten shallow, hyposaline soda lakes located in three different geographical regions in the Carpathian Basin (Central Europe) was characterized. These lakes, which frequently dry out completely, are extremely rich in PPP. Epifluorescence microscopy was applied to determine picocyanobacterial and picoeukaryotic cell abundance and PCR-based molecular techniques (denaturing gradient gel electrophoresis and cloning with phyllospecies delineation) to identify the members of PPP. Most of these lakes were eu- and hypertrophic with varying contribution of picocyanobacteria to the total PPP cell number. We found an unusually high PPP abundance with peaks of $8.16 \times 10^6$ cells mL$^{-1}$ for picoeukaryotes and $1.78 \times 10^7$ cells mL$^{-1}$ for picocyanobacteria. The majority of the retrieved PPP sequences belonged to picocyanobacteria (nonmarine Synechococcus/ Cyanobium), while others showed similarity to eukaryotic algal plastids (close to Trebouxia). Molecular analysis revealed significant genetic diversity in the PPP fraction of these lakes and showed that the closest relatives of our picocyanobacterial clones were recovered from different habitats, indicating seemingly no correlation between the 'saline' ecotypes and their phylogenetic position. Our results also confirmed that PPP might exploit different aquatic ecosystems and be successful even in the case of abrupt changes of environmental parameters (in our case, salinity). According to our knowledge, this is the first survey focusing on the identification of the PPP community members in turbid and alkaline lakes with extraordinarily high picoplankton productivity.

Key words: soda lake, photoautotrophic picoplankton, epifluorescence microscopy, PCR-based molecular techniques

1. INTRODUCTION

Shallow, turbid soda lakes are very characteristic of the Carpathian Basin. These are mostly intermittent shallow, alkaline pans that frequently dry out completely by the end of the summer. Their salinity varies from hypo- to mesosaline ranges in accordance with the season and water level (Schmidt & Fehér 2001; Schmidt 2003). Algological investigations of Hungarian soda lakes and Lake Fertő (Neusiedlersee) were intensive in the last century, which resulted in an exhaustive long list of species with limited information about pico-sized (<2 μm) algae (Dokulil & Padisák 1994; Padisák & Dokulil 1994; Schmidt & Fehér 2001). Recent studies of the photoautotrophic picoplankton (PPP) of these water bodies showed that red-fluorescent coccoid 1 μm-sized unicellular cyanobacteria and eukaryotic algae dominated (74-100%) the phytoplankton (Vörös & V.-Balogh 2003; Vörös et al. 2005).

According to the well-documented relationship in marine and freshwaters, the contribution of PPP to the total phytoplankton biomass decreases with the increase of the trophic state (Stockner 1988; Søndergaard 1991; Stockner & Shortreed 1991; Agawin et al. 2000; Bell & Kalff 2001; Callieri 2008), however the investigated hypertrophic Hungarian turbid pans do not follow this trend (Vörös & V.-Balogh 2003; Vörös et al. 2005).

Furthermore, the latest findings indicated that the soda lakes of the Carpathian Basin had the highest PPP abundance (both for picocyanobacteria and picoeukaryotic algae) ever reported in aquatic environments (Carrick & Schelske 1997; Vörös et al. 2005; Sarmento et al. 2008; Vörös et al. 2008; Somogyi et al. 2009).

Members of PPP can be enumerated by epifluorescence microscopy (e.g., Johnson & Sieburth 1979; Waterbury et al. 1979) or flow cytometry (e.g., Chisholm et al. 1988; Li & Wood 1988; Olson et al. 1990), but the identification of these bacterium-sized algae is often very problematic because of their small cell size and the limited number of distinct morphological characters. In many cases, these problems are associated with the known difficulties of cultivation (Ernst 1991; Rippka et al. 2000; Ernst et al. 2003), hence the species composition of picoplankton communities can mainly be defined with molecular methods. The application of culture independent techniques, such as cloning and sequence-based phyllospecies identification, fluorescent in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) provided new facilities in environmental microbiology, including the examination of aquatic microbial communities (reviewed by Dorigo et al. 2005). On the other hand, methods based on genetic characterization revised the taxonomy and systematics of picocyanobacteria (Honda et al. 1999; Turner et al. 1999; Robertson et al. 2001) and picoeukaryotic algae...
(Huss et al. 1999; Henley et al. 2004), and also revealed the presence of a significant uncultured fraction (Moon-van der Staay et al. 2001; Fuller et al. 2006; Ivanikova et al. 2007). The relatively large number of available small subunit ribosomal RNA gene sequences (SSU or 16S rDNA) for cyanobacteria and eukaryotic plastids (Cole et al. 2003) provides a considerable database for oxygenic phototrophs in the course of DNA-based community diversity investigations.

In this study, we examined the PPP community inhabiting the extremely turbid and productive lakes of the Carpathian Basin with the application of epifluorescent enumeration and molecular characterization.

2. MATERIALS AND METHODS

2.1. Study sites and sampling

Samples were collected between 2003 and 2005 from ten turbid, shallow water bodies of the Carpathian Basin from three different geographical regions: the Fertő-Hanság Region (Lake Fertő), the Vojvodina Region (Lake Rusanda and Slano Kopovo) and the Kiskunság Region (Fig. 1, Tabs 1 and 2).

Lake Fertő (Neusiedlersee) is a large, turbid, shallow lake on the Austrian-Hungarian border. The reed-zone divides the open water into several parts: there are large, open water areas and small, isolated inner ponds. Our sample was taken from the turbid, white-colored open-water area in the Hungarian part of the lake. The lake is characterized as meso-eutrophic (Dokulil & Padisák 1994), and chlorophyll-a maximum usually does not exceed 25 µg L⁻¹.

Kastély Pond is a man-made shallow pond in the Kiskunság Region of South Hungary, which does not have typical turbid, white-colored water due to its small surface area and the relatively large water depth. The other investigated lakes are typical turbid, intermittent, shallow pans with white-colored water and very low Secchi-disk (SD) transparency (1-5 cm), due to the high concentration of suspended solids and dissolved organic substances (Vörös et al. 2006). The water is rich in Na⁺, HCO₃⁻, SO₄²⁻, and Cl⁻ ions with pH values between 9 and 10 (Tab. 1). Most lakes of the Kiskunság Region could be characterized as hypertrophic water bodies (Vörös & V.-Balogh 2003; Vörös et al. 2005; Vörös et al. 2008; Somogyi et al. 2009). The trophic state of three lakes (Lake Slano Kopovo, Lake Rusanda and Lake Kis-Sós) could not be determined from our single measurement.

Temperature, pH and conductivity values of each sample were determined with a MultiLine P4 meter (WTW, Weilheim, Germany).

Surface-water samples were collected for algological and molecular characterization, and the samples were transferred to the laboratory without preservation in a thermo box in dark conditions. Sample processing started within 3-6 hours after sampling.

2.2. Phytoplankton biomass and PPP composition

Chlorophyll-a concentration of the phytoplankton was determined from fresh samples. Aliquots (10–100 mL depending on algal biomass) were filtered through GF-5 glass fiber filters. Chlorophyll-a was extracted with hot methanol (64.7 °C, 1 min) and the concentra-
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tion was determined spectrofluorimetrically according to Wetzel & Likens (1991).

The abundance and composition of the PPP was determined from fresh, unpreserved samples. Aliquots of 0.5-3 mL were filtered through black polycarbonate filters with 0.4 µm pore-size. The filters were placed on microscopic slides and were embedded into 50% glycerol. The slides were examined with an Optiphot 2 epifluorescence microscope (Nikon, Japan) with 1000× magnification using blue-violet (BV-2A) and green (G-2A) excitation light. Following the routine enumeration protocol for identifying PPP types (picocyanobacteria and picoeukaryotic algae), first the picophytoplankton cells were located under blue-violet excitation. Picoeukaryotes show deep red fluorescence under this excitation due to their chlorophyll-α content. Phycoerythrin-rich picocyanobacteria fluorescence is bright yellow-orange, while phycocyanin-rich picocyanobacteria show only weak red autofluorescence. By switching to green excitation for the same field, picoeukaryotic cells do not (or just very weakly) show autofluorescence. The main property distinguishing picoeukaryotic algae and phycoerythrin-rich picocyanobacteria under epifluorescence microscope is the presence of phycobiliproteins in cyanobacteria, which show greatly enhanced (red) autofluorescence under green excitation (MacIsaac &

<table>
<thead>
<tr>
<th>Lake</th>
<th>Sampling date</th>
<th>Temperature (°C)</th>
<th>Conductivity (µS cm⁻¹)</th>
<th>Chlorophyll-α (µg L⁻¹)</th>
<th>Abundance of picocyanobacteria (10⁴ cells mL⁻¹)</th>
<th>Abundance of picoeukaryotes (10⁴ cells mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Fertő</td>
<td>28 Apr 2004</td>
<td>16</td>
<td>2500</td>
<td>31</td>
<td>309 &lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Lake Slano Kopovo</td>
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<td>15</td>
<td>3220</td>
<td>6.0</td>
<td>168 3.7</td>
<td></td>
</tr>
<tr>
<td>Lake Rusanda</td>
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<td>15</td>
<td>10400</td>
<td>2.0</td>
<td>7.3 0.5</td>
<td></td>
</tr>
<tr>
<td>Lake Kis-Sós</td>
<td>23 Apr 2005</td>
<td>11</td>
<td>1845</td>
<td>3.0</td>
<td>0.1 8.6</td>
<td></td>
</tr>
<tr>
<td>Kastély Pond</td>
<td>24 Jul 2004</td>
<td>28</td>
<td>5220</td>
<td>32</td>
<td>356 &lt;0.1</td>
<td></td>
</tr>
<tr>
<td>1 May 2005</td>
<td>19</td>
<td>4670</td>
<td>24</td>
<td>399 &lt;0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kelemen-szék Pan</td>
<td>17 Oct 2004</td>
<td>9</td>
<td>7950</td>
<td>23</td>
<td>10 42</td>
<td></td>
</tr>
<tr>
<td>7 Jul 2005</td>
<td>28</td>
<td>6300</td>
<td>7.6</td>
<td>32 12.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 Apr 2004</td>
<td>12</td>
<td>4100</td>
<td>9.0</td>
<td>0.1 33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Jul 2004</td>
<td>30</td>
<td>9050</td>
<td>81</td>
<td>1032 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 Oct 2004</td>
<td>8</td>
<td>16480</td>
<td>57</td>
<td>623 107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 Apr 2005</td>
<td>16</td>
<td>6710</td>
<td>120</td>
<td>213 171</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>28</td>
<td>9700</td>
<td>81</td>
<td>519 65</td>
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<tr>
<td>3 Sep 2004</td>
<td>24</td>
<td>4410</td>
<td>44</td>
<td>22.7 16.6</td>
<td></td>
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<tr>
<td>17 Oct 2004</td>
<td>11</td>
<td>4700</td>
<td>34</td>
<td>107 816</td>
<td></td>
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</tr>
<tr>
<td>7 Jul 2005</td>
<td>29</td>
<td>3700</td>
<td>11</td>
<td>30 34</td>
<td></td>
<td></td>
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<tr>
<td>Lake Fehér</td>
<td>27 May 2004</td>
<td>21</td>
<td>3190</td>
<td>81</td>
<td>0.1 210</td>
<td></td>
</tr>
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<td>1 May 2003</td>
<td>19</td>
<td>4150</td>
<td>30</td>
<td>928 &lt;0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Jun 2003*</td>
<td>21</td>
<td>13890</td>
<td>120</td>
<td>23 39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zab-szék Pan</td>
<td>15 Sep 2004</td>
<td>24</td>
<td>5160</td>
<td>11</td>
<td>461 73</td>
<td></td>
</tr>
<tr>
<td>17 Oct 2004</td>
<td>8</td>
<td>6640</td>
<td>27</td>
<td>2.1 58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Jul 2005</td>
<td>28</td>
<td>4100</td>
<td>14</td>
<td>36 19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tab. 1. Major characteristic features of the sampled soda lakes in the Carpathian Basin (based on Vörös et al. 2006). Lake numbers refer to codes on figure 1. *: based on the values measured on sampling dates.

<table>
<thead>
<tr>
<th>Region (Country)</th>
<th>Lake</th>
<th>Geographical coordinates</th>
<th>Surface area (km²)</th>
<th>Water depth (cm)</th>
<th>SD transparency (cm)</th>
<th>pH</th>
<th>Dominant cation</th>
<th>Dominant anion(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertő-Hanság (Hungary-Austria)</td>
<td>1 Lake Fertő (Neusiedlersee)</td>
<td>47°40' 16°45'</td>
<td>300</td>
<td>100-120</td>
<td>5-30</td>
<td>9.0-9.3</td>
<td>Na⁺</td>
<td>HCO₃⁻&gt;SO₄²⁻</td>
</tr>
<tr>
<td>2 Lake Slano Kopovo*</td>
<td>45°37' 20°12'</td>
<td>1.04</td>
<td>30</td>
<td>2</td>
<td>8.9</td>
<td>Na⁺</td>
<td>Cl⁻&gt; SO₄²⁻&gt;HCO₃⁻</td>
<td></td>
</tr>
<tr>
<td>3 Lake Rusanda*</td>
<td>45°31' 20°17'</td>
<td>1.70</td>
<td>20</td>
<td>4</td>
<td>9.3</td>
<td>Na⁺</td>
<td>SO₄²⁻&gt; HCO₃⁻&gt;Cl⁻</td>
<td></td>
</tr>
<tr>
<td>4 Lake Kis-Sós*</td>
<td>46°44' 19°59'</td>
<td>0.10</td>
<td>22</td>
<td>5</td>
<td>9.1</td>
<td>Na⁺</td>
<td>HCO₃⁻</td>
<td></td>
</tr>
<tr>
<td>5 Kastély pond</td>
<td>46°40' 19°08'</td>
<td>0.01</td>
<td>60-100</td>
<td>17-35</td>
<td>9.0-9.9</td>
<td>Na⁺</td>
<td>HCO₃⁻&gt;Cl⁻</td>
<td></td>
</tr>
<tr>
<td>6 Kelemen-szék pan</td>
<td>46°49' 19°11'</td>
<td>1.20</td>
<td>&lt;50</td>
<td>1-5</td>
<td>9.0-9.7</td>
<td>Na⁺</td>
<td>HCO₃⁻&gt;Cl⁻</td>
<td></td>
</tr>
<tr>
<td>7 Bödő-szék pan</td>
<td>46°46' 19°08'</td>
<td>1.17</td>
<td>&lt;50</td>
<td>1-5</td>
<td>9.0-9.7</td>
<td>Na⁺</td>
<td>HCO₃⁻&gt;Cl⁻</td>
<td></td>
</tr>
<tr>
<td>8 Bödő-szék pan</td>
<td>46°33' 20°02'</td>
<td>0.50</td>
<td>&lt;50</td>
<td>1-5</td>
<td>9.0-9.7</td>
<td>Na⁺</td>
<td>HCO₃⁻&gt;Cl⁻</td>
<td></td>
</tr>
<tr>
<td>9 Lake Fehér</td>
<td>46°28' 20°37'</td>
<td>0.70</td>
<td>&lt;50</td>
<td>1-5</td>
<td>9.1-9.8</td>
<td>Na⁺</td>
<td>HCO₃⁻&gt;Cl⁻</td>
<td></td>
</tr>
</tbody>
</table>

Tab. 2. List of investigated samples with their phytoplankton biomass and PPP abundance collected from different soda lakes in the Carpathian Basin. *: sample was cloned.
Stockner 1993). At least 20 fields were photographed with a Spot RT color camera, and the PPP were counted on these pictures to avoid fluorescence fading. In all cases, a minimum of 400 cells were counted with an error of 10% (Lund et al. 1958).

2.3. DNA extraction and amplification

After the concentration of 50-100 mL water sample by centrifugation (5000 g, 10 min), pellets were stored at -20 °C for DNA extraction. First, 0.6 mL CLS-Y (Bio101 Systems, La Jolla, CA, USA), 300 mg glass bead and 10 mg of polyvinyl-polypyrrolidone were added to the samples, then the cells were disrupted in a Mixer Mill MM301 (Retsch, Haan, Germany) (1 min, 30/s). Extraction of the genomic DNA was carried out with the Bacterial Genomic DNA Mini-Prep Kit (V-Fermentas, Vilnius, Lithuania), 1X PCR buffer (Fermentas, Vilnius, Lithuania), 1X PCR buffer (Fermentas, Vilnius, Lithuania), 1X PCR buffer (Fermentas, Vilnius, Lithuania), 0.325 μM of 27F and 1492R primers (Lane 1991) (Tab. 3), and the following temperature profile: initial denaturation at 98 °C for 5 min, followed by 35 cycles of 30 sec at 94 °C (denaturation), 30 sec at 52 °C (annealing), 1 min at 72 °C (extension), and final extension at 72 °C for 10 min. A second PCR (nested PCR) with Cyanobacteria-specific primers was performed to increase yield and achieve specificity. The composition of the nested PCR was similar to the above described protocol with the exception of the use of CYA359F (without GC-clamp) and CYA781R primers and the clones were grouped according to their ARDRA (Amplified Ribosomal DNA Restriction Analysis) patterns generated (13890 µS cm⁻¹) was selected for cloning.

The purified PCR product was cloned using the TOPO TA Cloning® Kit containing pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The inserted 16S rDNA fragments recovered from DGGE bands were reamplified with the CYA359 (without GC-clamp) and CYA781R primers using the same PCR conditions as applied prior to DGGE analysis.

2.5. Cloning

A sample (6 June, 2003, Zab-szék Pan) with high chlorophyll-a content (120 µg L⁻¹) and high conductivity (13890 µS cm⁻¹) was selected for cloning.

The purified PCR product was cloned using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a Model 310 Genetic Analyzer (Applied Biosystems). Sequences were analyzed using the DNA Sequencing Analysis Software v5.2 (Applied Biosystems). The chroma-
tograms were corrected manually, and primer sequences were removed using the Chromas software v1.45 (Technelysium Pty Ltd, Australia). The generated sequences were compared to the GenBank nucleotide database using the Blast program (Altschul et al. 1997). In the case of non-oxygenic phototrophic bacterial sequences, identification was enhanced with a search for type strains using EzTaxon (Chun et al. 2007). The neighbor-joining (Saitou & Nei 1987) phylogenetic tree inferred from 301 unambiguously aligned nucleotide positions of the 16S rDNA was constructed with the MEGA4 software (Tamura et al. 2004) using ClustalW alignment (Thompson et al. 1994).

Partial 16S rDNA sequences obtained in this study have been submitted to GenBank under the following accession numbers: EU546171-EU546198 (DGGE, 28 sequences) and EU647634-EU647645 (cloning, twelve sequences).

3. RESULTS

3.1. Phytoplankton biomass and PPP composition

The studied water bodies could all be considered as brackish (hyposaline) aquatic systems, since the measured conductivity values amounted to 1.0-9.5‰ salinity, and most of these turbid, saline lakes had a relatively high chlorophyll-α content (Tab. 2), indicating a high trophic state based on the OECD classification system (OECD 1982). In Lake Fertő, the chlorophyll-α concentration was 31 μg L⁻¹, in accordance with its known meso-eutrophic state. The saline lakes of Vojvodina (Lake Slano Kopovo and Lake Rusanda) and one lake from the Kiskunság Region (Lake Kis-Sós) had relatively low chlorophyll-α concentration at sampling (6.0, 2.0 and 3.0 μg L⁻¹, respectively). Kastély Pond, Bödönszék Pan, Lake Fehér and Zab-szék Pan had maximum values of chlorophyll-α concentration exceeding the 75 μg L⁻¹ threshold defined for the hypertrophic state. In the samples taken from Kelemen-szék and Bödönszék Pans, this value was lower (7.6-24 and 11-44 μg L⁻¹, respectively).

The PPP was dominated by eukaryotic picoalgae and phycocyanin-rich picocyanobacteria (Tab. 2). Phycoerythrin-rich picocyanobacteria were not detected in our samples. PPP density varied from 7.8 × 10⁴ to 1.82 × 10⁷ cells mL⁻¹, the highest cell numbers were measured in Bödőszék Pan. The abundance of picocyanobacteria varied from 0.1 × 10⁴ to 1.78 × 10⁷ cells mL⁻¹, the maximum value was measured in Bödőszék Pan in September, 2004. The abundance of picoeukaryotes varied from <0.1 × 10⁴ to 8.16 × 10⁶ cells mL⁻¹, the maximum value was measured in Bödőszék Pan in October, 2004.

3.2. Molecular characterization of the PPP community

The DGGE analysis revealed a variable pattern with notable differences among the samples (Fig. 2). Sequence analysis of the 28 distinct DGGE bands showed that all reamplified sequences were related to either cyanobacterial or eukaryotic plastid 16S rDNA (Fig. 3).
Fig. 3. Phylogenetic position to their closest relatives of the sequences related to oxygenic phototrophs determined in this study. Bootstrap values <50% are not shown. Bar indicates 0.01 nucleotide substitution per site. Isolation details are also shown for environmental clones and picocyanobacterial isolates. Sequences determined in this study appear in bold letters.
The twelve different ARDRA types resulted from the restriction endonuclease screening of the Zab-szék Pan clone library consisting of 76 clones. Sequence analysis of the representative clones (Fig. 3, Tab. 4) showed that 44.7% of the clone library was related to sequences derived from oxygenic phototrophs (31.5% to picocyanobacteria and 13.2% to plastid sequences). The remaining sequences (55.3% of total clones) showed low similarity values to the bacterial phylum Verrucomicrobia.

PP sequences from the Zab-szék Pan clone library belonged to three distinct branches within the picophytoplankton clade of Cyanobacteria (sensu Urbach et al. 1998). Picocyanobacterial sequences from the other samples also had some genetic diversity (Lake Rusanda, 23 April, 2005: 98.0-99.0%; Lake Kis-Sös, 23 April, 2005: 97.6-100% pairwise similarities) or were almost or completely identical (Böddi-szék Pan, 3 September, 2004: 99.7%; Lake Slano Kopovo, 23 April, 2005: 99.3-100% pairwise similarities; sequences derived from Lake Fehér and Lake Ferto were identical, respectively). When samples of different time periods of the same lake were compared, picocyanobacteria could be affiliated to at least two different phylogenetic groups (Fig. 3). Two clones (DGGE clone 2 and 3) originating from the same sample (Kastély Pond, 4 July, 2004) were related to the non-PPP cyanobacterial genus Anaabaenopsis (Nostocales), both sharing >99% similarity with the PCC9215 strain. The presence of this genus in the sample was also observed during microscopic investigation.

Sequences related to eukaryotic algal plastids formed two separate groups, both within the family of Trebouxiophyceae, closely related to the genera Chlorella and Koliella.

Nonetheless, our molecular identification was based on a relatively short fragment of the 16S rDNA, previously undiscovered members of PPP were identified in the investigated soda lakes (e.g., the Trebouxiophycean clone Z0306c8 or the picocyanobacterial group formed by clones Z0306c47 and DGGE clone 28 in Fig. 3).

### 4. DISCUSSION

#### 4.1. Occurrence of PPP in the alkaline lakes of the Carpathian Basin

The investigated water bodies were highly productive and characterized as eu- or hypertrophic, as it was confirmed in most cases with our punctual sampling. The highest chlorophyll-a concentration was detected in two soda lakes of the Kiskunság Region, in Böddi-szék and in Zab-szék Pans (120 µg L⁻¹). But the maximum measured chlorophyll-a concentration was much higher in the lakes of this region: 300 µg L⁻¹ in Kelemen-szék Pan and 797 µg L⁻¹ in Bődös-szék Pan (Somogyi et al. 2009).

A high number of PPP cells was detected in the investigated turbid, alkaline lakes, ranging from 10⁵ to 10⁷ cells mL⁻¹. In extremely productive periods, the abundance of picoeukaryotic and picocyanobacterial cells could be even higher in the lakes of the Kiskunság Region. Maximum observed values were 1.08 × 10⁸ cells mL⁻¹ (Somogyi et al. 2009) and 1.03 × 10⁸ cells mL⁻¹ (Vörös et al. 2005), respectively. These values were the highest ever reported in the literature (Sarmiento et al. 2008; Somogyi et al. 2009). The extremely high cell abundance of planktonic phototrophs is supposedly related to the fact that there is no nutrient limitation in these water bodies due to the high nitrogen- and phosphorous-load from wintering and migrating aquatic birds (Boros et al. 2008). The increased surface-to-volume ratio of cells that is hypothesized to be affiliated with better light utilization (Agustí 1991) and nutrient uptake efficiency (Reynolds 2006) could account for the selective advantage of this pico-sized fraction in such turbid, light limited environments with high nutrient supply.

Picoeukaryotic algae and phycocyanin-rich picocyanobacteria constituted the PPP community of the investigated saline lakes, but picocyanobacteria with phycocerythrin pigment dominance were absent from all samples, in line with previous studies (Vörös & V.-

<table>
<thead>
<tr>
<th>Sequenced clone</th>
<th>Percentage of clone library</th>
<th>Sequence similarity</th>
<th>Closest relative</th>
</tr>
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<tbody>
<tr>
<td>Z0306c1</td>
<td>11.8%</td>
<td>99.0%</td>
<td>clone LS40 (Lake Superior)</td>
</tr>
<tr>
<td>Z0306c3</td>
<td>2.6%</td>
<td>88.2%</td>
<td>Opitutus terrae PB90-1T</td>
</tr>
<tr>
<td>Z0306c5</td>
<td>6.6%</td>
<td>78.8%</td>
<td>Sphingomonas koreensis KCTC2882T</td>
</tr>
<tr>
<td>Z0306c7</td>
<td>2.6%</td>
<td>90.6%</td>
<td>Opitutus terrae PB90-1T</td>
</tr>
<tr>
<td>Z0306c8</td>
<td>13.2%</td>
<td>94.7%</td>
<td>Chlorella kessleri SA211-11g, plastid</td>
</tr>
<tr>
<td>Z0306c9</td>
<td>15.8%</td>
<td>90.9%</td>
<td>Opitutus terrae PB90-1T</td>
</tr>
<tr>
<td>Z0306c11</td>
<td>15.8%</td>
<td>87.9%</td>
<td>Verrucomicrobium spinosum DSM4136T</td>
</tr>
<tr>
<td>Z0306c13</td>
<td>18.4%</td>
<td>99.2%</td>
<td>Cyanobium sp. JMM10D4</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>2.6%</td>
<td>86.1%</td>
<td>Rubritalea squamosifacies DSM18772T</td>
</tr>
<tr>
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<td>1.3%</td>
<td>97.8%</td>
<td>Synechococcus sp. PCC9005</td>
</tr>
</tbody>
</table>

Tab. 4. Phyllospecies identification results based on cloning the water sample from Zab-szék Pan taken on 6 June, 2003. Type strain.
saline lakes were related to the picophytoplankton clade.

4.2. Phylogenetic position of PPP in the alkaline lakes

The retrieved PPP sequences from the investigated saline lakes were related to the picophytoplankton clade of Cyanobacteria (sensu Urbach et al. 1998) or to *Chlorella* species within the family Trebouxiophyceae (Chlorophyta). These are the most abundant members of the pro- and eukaryotic PPP in freshwater ecosystems (Callieri 2008). There is only limited information available regarding the taxonomic position of PPP inhabiting the turbid, alkaline lakes of the Carpathian Basin. Somogyi et al. (2009) isolated and identified one picoeukaryotic and one picocyanobacterial strain from Bődöi-szék Pan (Kiskunság Region). On the basis of the partial sequence analysis of the 16S rDNA, the picocyanobacterial strain (ACT0616) was identified as a nonmarine member of the picophytoplankton clade, sharing 98.7% pairwise similarity with our DGGE clone 7 (Fig. 3). On the basis of the partial analysis of the 18S rDNA, the picoeukaryotic strain was distantly related to other algal isolates, and therefore this strain was proposed to be a member of a candidate new chlorophyte genus (B. Somogyi, unpublished results). Considering the selectivity of classical isolation techniques (Ernst 1991; Ernst et al. 2003), the composition of the PPP could not be defined with the identification of a few isolated strains, therefore this can be regarded as the first consistent survey dealing with the taxonomic characterization of the PPP community composition in the turbid, alkaline water bodies of the Carpathian Basin.

A relatively high genetic diversity was found among the sequences determined in this study, especially in case of picocyanobacteria. We suppose that all picocyanobacterial sequences recovered from these lakes could be derived from phycocyanin-rich cells, since no phycoerythrin-rich cells were observed during the microscopic investigations.

Picocyanobacterial sequences from Lake Fertő belonged to the *Cyanohabium gracile* cluster (sensu Ernst et al. 2003), a group containing both phycoerythrin- and phycocyanin-rich isolates from different habitats and geographical locations. PPP sequences from the Kiskunság soda lakes had high genetic variance and did not diverge from the sequences of the Vojvodina Region: no geographical separation was observed in the phylogenetic tree. The closest relatives of almost all picocyanobacterial sequences identified in this study were recovered from distant geographical regions and/or from different habitats (lake or river, brackish or marine environment).

The retrieved sequences were widely distributed within the picophytoplankton clade; in other words, there seems to be no correlation between the 'saline' ecotypes of picocyanobacteria and their phylogenetic position, since strains or clones derived from brackish and marine environments clustered with freshwater sequences (Fig. 3, Crosbie et al. 2003; Budinoff & Hollibaugh 2007; Sánchez-Baracaldo et al. 2008). Recent molecular investigations did not only provide information about the widespread geographical distribution of many genotypes, but also revealed that freshwa-
ter/terrestrial picocyanobacterial communities shared much greater diversity than their marine counterparts (this study; Crosbie et al. 2003; Ivanikova et al. 2007; Sánchez-Baracaldo et al. 2008). This could be explained with a more rapid speciation induced by geographical barriers or with the long evolutionary history of freshwater picocyanobacteria (Sánchez-Baracaldo et al. 2008). The latter was supported with a study combining phylogenetic analysis of slowly evolving genes and morphological characters (Sánchez-Baracaldo et al. 2005), which demonstrated the probable terrestrial/freshwater origin of Cyanobacteria coupled with small cell diameter and free-living planktonic habit (a typical Synechococcus) shared in these ancient lineages. The potential to colonize marine environments, as well as the acquisition of derived traits (complex morphology, thermophily, motility, etc.) were gained later in the independent cyanobacterial lineages.

Although most nonmarine picocyanobacterial clusters appear to be cosmopolitan, intensive investigations on PPP communities gave rise to new groups with the restriction of some genotypes to a particular ecosystem or geographical location (Crosbie et al. 2003; Ivanikova et al. 2007; Sánchez-Baracaldo et al. 2008). The adaptive potential of PPP to extreme environmental conditions (e.g., in our case high pH, salinity and light limitation) also illustrates the evolutionary success of these small-sized phototrophs.

4.3. Remarks on the biases associated with the methods applied in this study

With regards to the DGGE technique, we noticed that identical sequences could generate multiple bands (e.g., DGGE clones from Lake Fertő), and sequences reamplified from excised bands located in the same position of the gel could be different (e.g., DGGE clones 11-14). One possible explanation for obtaining identical sequences from different bands in the same lane is the occurrence of both homoduplex and heteroduplex molecules (Ferris & Ward 1997). The co-migration of different fragments was also reported (Sekiguchi et al. 2001), but other deviations may also appear and influence the results (Kisand & Wikner 2003; Nikolausz et al. 2005).

The extent to which the observed fine-scale variation of 16S rDNA sequences could be related to artefacts (e.g., Taq errors, heteroduplex molecules), heterogeneity among paralogous operons or to the co-existence of closely-related taxa is also uncertain (Acinas et al. 2004).

A notable portion of our clone sequences were related to Verrucomicrobia. The members of this phylum are well known inhabitants of eutrophic or extreme environments such as soda lakes (Schlesner et al. 2006). Interestingly, sequences related to microorganisms other than oxygenic phototrophs were not retrieved by DGGE. This could be the result of the different specificity of the forward primers applied for cloning (CYA106F) and for DGGE (CYA359F) that resulted in the disparate amplification of taxa (Sipos et al. 2007). Similar problems of unspecific amplification associated with the primers applied in this study for cloning were also reported (Katano et al. 2001; Ivanikova et al. 2007).

These phenomena draw attention to the biases associated with molecular methods that use complex PCR products and to question the application of DGGE pattern comparison or pattern analysis without sequencing.

5. CONCLUSIONS

This study characterized PPP communities of ten soda lakes located in the Carpathian Basin. In some of these water bodies, their abundance could reach the highest values ever reported in literature with exclusive dominance of PPP in total primary production. The contribution of picoeukaryotes to the total PPP was highly variable among lakes, ranging from 0 to 100%. The development of extreme hypertrophy was related to the high nutrient concentration.

Despite the hyposaline character of these lakes, the prokaryotic members of the PPP were phylogenetically related to the nonmarine Synechococcus/Cyanobium group within the picophytoplankton clade, while the eukaryotic members were affiliated to Chlorella isolates. Most of our sequences were related to clones or strains originating from distant geographical locations that supported the widespread dispersal of some groups of PPP. The relatively high genetic diversity of PPP in such extreme environments with rapidly changing physicochemical parameters (especially in the case of picocyanobacteria) demonstrates the adaptive potential and confirms the evolutionary success of these small-sized phototrophs.

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